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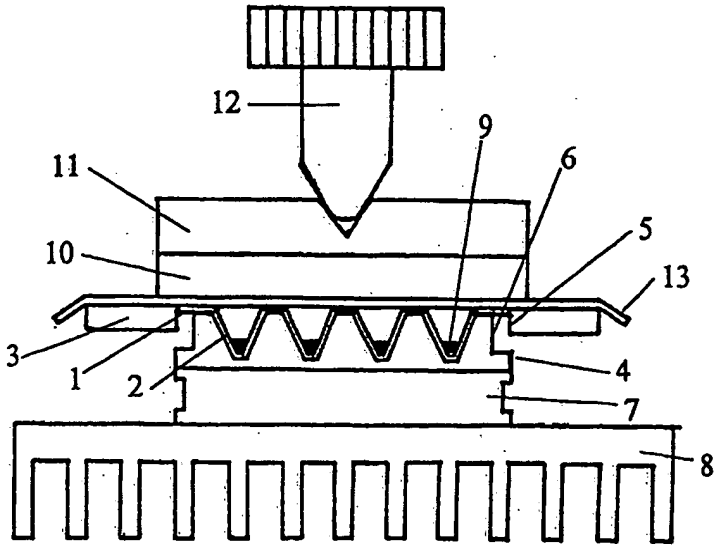

(57) Abstract

Ultrathin-walled multiwell reactors for heat block thermocycling of samples comprising an array of small-volume wells of identical height with similarly shaped sample wells formed in the top surface of the heat block of the thermocycler are provided. The multiwell plates are preferentially vacuumformed out of a 30-50 micron thick thermoplastic film and can be used for rapid, oil-free temperature cycling of small (1-10μl) volume samples.

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Ultrathin-walled multiwell plate for heat block thermocycling

The invention relates to plastic plates for conventional heat block thermocycling of biological samples, particularly to multiwell plates. More specifically, it relates to ultrathin-walled multiwell plates with an improved heat transfer to small-volume samples. Such plates can be used for rapid temperature cycling of multiple, small-volume samples (i.e. 1-20 µl) by using heat block thermocyclers with an increased block temperature ramping rate (i.e. 4° C/second and greater) and standard heated-lid technology for sealing the samples.

Temperature cycling of biological samples is a central moment in DNA amplification by the polymerase chain reaction (PCR) (Saiki et al., Science, 239, 487-491 [1988]). Much effort is being expended in developing various alternative reactors and technologies for rapid temperature cycling of small-volume samples (Kopp et al., Science 280, 1046-1048 [1998]; Belgrader et al., J.Forensic Science 43, 315-319 [1998]; Wittwer et al., Analytical Biochem., 186, 328-331 [1990] and U.S. Patent No 5,455,175; Woolley et al., Analytical Chem., 68, 4081-4086 [(1996]).

One commercially available type of microreactor and thermocycler for rapid temperature cycling of small samples is a glass capillary tube and a hot-air thermocycler from Roche Molecular Biochemicals (cat No. 1909 339 and cat No. 2011468, respectively). The glass capillary tube can hold reaction volumes ranging from 10 to 20 µl. The hot-air thermocycler can hold 32 capillaries and perform 30 - 40 PCR cycles in 20-30 minutes. However, these rapid DNA amplification technology is connected with various disadvantages, for example:

a) The handling of the individual capillaries is relatively cumbersome.

b) The relatively large glass surface adsorbs components of the standard PCR-mixtures. This might inactivate the reaction. Therefore, various carrier molecules, i.e. proteins or even DNA, must be added and the concentrations of the components reoptimized.

c) The cost of the capillary tube, as a disposable PCR container, is high when compared to the standard 0.2 ml PCR tube.

d) The experimental throughput using this system is limited.

It is surprising that only little research has been conducted to improve the basic performance in sample size and speed of the widely used, conventional heat block

thermocycling of samples contained in plastic tubes or multiwell plates. One known improvement of heat block temperature cycling of samples contained in plastic tubes has been described by Half et al. (Biotechniques, 10, 106-112, [1991] and U.S. Patent No 5,475,610). They describe a special PCR reaction-compatible one-piece plastic microcentrifuge tube, i.e. a thin-walled PCR tube. The tube has a cylindrically shaped upper wall section, a relatively thin (i.e. approximately 0.3 mm) conically-shaped lower wall section and a dome-shaped bottom. The samples as small as 20 µl are placed into the tubes, the tubes are closed by deformable, gas-tight caps and positioned into similarly shaped conical wells machined in the body of the heat block. The heated cover compresses each cap and forces each tube down firmly into its own well. The heated platen (i.e. heated lid) serves several goals by supplying the appropriate pressure to the caps of the tubes: it maintains the conically shaped walls in close thermal contact with the body of the block; it prevents the opening of the caps by increased air pressure arising in the tubes at elevated temperatures. In addition, it maintains the parts of the tubes that project above the top surface of the block at 95° -100° C in order to prevent water condensation and sample loss in the course of thermocycling. This made it possible to exclude the placing of mineral oil or glycerol into the wells of the block in order to improve the heat transfer to the tubes and the overlaying of the samples by mineral oil that prevented evaporation but also served as added thermal mass. In addition, the PCR tubes can be put in a two-piece holder (US patent 5,710,381) of an 8x12, 96-well microplate format, which can be used to support the high sample throughput needs with any number between 1 and 96 individual reaction tubes.

In DE 4022792 the inventors describe a plate with cylindrically shaped walls of the wells and spherically shaped bottoms thereof. The individual wells of the plate were formed by melting a polycarbonate sheet in the range of 0.27-0.5 mm by a stream of hot air. This technology leads to relatively thin walls in the range of 0.08-0.2mm. The biological samples were placed into the wells, covered with polycarbonate film (0.1 mm) and the individual wells were thermosealed by a special press. Upon sealing the plate was placed on the thermoblock and fixed by screws. Though theoretically the heat transfer to the samples is improved, however, the way of positioning the plate on the block and the cylindrical and spherical geometry of the well prevent a close thermal contact with the heating block. During thermocyling, due to the large thermal expansion, the plate fixed by

screws becomes deformed and the close thermal contact is not maintained anymore. Therefore, by using the above technology rapid cycling reactions cannot be performed.

The other known improvement of heat block thermocycling is described in PCT patent application WO 98/43740. It concerns a heat block thermocycler with an increased ramping rate, i.e. 4° C/second). The thermocycler can hold 96 PCR tubes (each of a volume of 0.2 ml) or 96-well PCR plates. Theoretically, the thermocycler can perform 30 PCR cycles in 20-30 minutes, provided that only a few seconds are spent to reach the temperature equilibrium between the heat block and the samples.

However, as described in U.S. Patent No 5,508,197, even if the temperature of the heat-transfer media, i.e. water, is changed almost instantaneously, it takes approximately 15 seconds to reach equilibrium between water and the 15-20 µl samples in the standard PCR plates. This means that for 30 PCR cycles approximately 20 minutes are spent to reach the equilibrium between heat-transfer media and the 15-20 µl samples in the plates.

In comparison, the above mentioned heat block cycler (WO 98/43740) operating at a ramping rate of 4° C/second, needs for the heat-block temperature transitions during 30 PCR cycles 10 minutes only. This shows that the major limiting factor for rapid temperature cycling of small samples in platic PCR tubes or PCR plates is the low efficiency of the heat transfer through the walls of conventional PCR tubes or plates, respectively.

The present invention concerns plastic multiwell plates for performing heat block thermocycling of multiple samples. More specifically, it concerns ultrathin-walled multiwell plates with an improved heat transfer to small samples. Ultrathin-walled multiwell plates are suited for rapid, oil-free, heat block temperature cycling of small-volume samples (i.e. approximately 1-20 µl), whereas the lower limit is given by the reliability of the conventional pipetting systems.

Figure 1 illustrates an example of a multiwell plate according to the invention.

Figure 2 illustrates the positioning of the plate in the block of the thermal cycler.

One aspect of the present invention concerns the considerably decreased thickness (i.e. approximately 7.5-15 fold) of the well walls when compared to known thin-walled PCR tubes (U.S. Patent No 5,475,610). This can be reached, for example, by means of thermoforming the plates out of thin thermoplastic films. Such thermoplastic films are, for example, polyolefin films, such as metallocene-catalyzed polyolefin films and/or copolymer films. Usually, the multiwell plate is vacuumformed out of cast, unoriented

polypropylene film, polypropylene-polyethylene copolymer films or metallocene-catalyzed polypropylene films. The film is formed into a negative ("female") mould comprising a plurality of spaced-apart, conically shaped wells which are machined in the body of a mould in the shape of rectangular- or square-array. The thickness of the film for vacuumforming conically shaped wells is chosen according to the standard rule used for thermoforming, i.e. thickness of the film = well draw ratio x thickness of the wall of the formed well.

For example, vacuumforming wells with a draw ratio of two and an average thickness of the walls of 30 microns results in a film thickness of 60 microns. The average optimum wall thickness was found to be 20-40 microns. The thickness of the well is reduced 7.5-15 fold when compared to the wall thickness of the formerly improved PCR tube desribed in U.S. Patent No 5,475,610. Using the Fourier equation for heat transfer and the equation for temperature transfer through solid substances, it can be shown that heat transfer through one square millimeter of the surface of the well of the plate is increased 7.5-15 fold and the time of temperature transfer through the wall is decreased 56-225 fold when compared to the said PCR tube. This drastic decrease in time can be explained by the fact that the time needed for the transfer of temperature front is proportional to the square power of distance. It can be easily calculated that the time of the temperature transfer through the ultrathin walls of the multi-well plate is in the range of milliseconds, whereas for the said PCR tube (U.S. Patent No 5,475,610) it is in the range of seconds. This explains the well known fact that thin (20-40 microns) plastic films are poor thermo insulators.

The thickness of the walls of the formed wells is gradually reduced to the bottoms of the wells due to vacuumforming of the wells into a negative mould. This geometry of the walls of the wells provides several advantages:

- The relatively thick upper parts of the walls of the wells cause additional rigidity of the whole multiwell plate.
- During heating of the heat block of the thermocycler, a vertical temperature gradient is formed in the sample, due to the gradient of the well-wall thickness. This vertical temperature gradient causes intensive convective mixing of the sample in conically shaped wells and increases the heat transfer through the sample. In comparison, this convective mixing of the sample is much less efficient in conventional PCR plates/tubes with a uniform wall thickness.

Another aspect of the invention concerns the height of the wells of the multiwell plate. The height of the conically shaped wells is equal to the height of the similarly shaped sample wells machined in the body of the heat block. Thus, this geometry of the wells (2) enables the positioning of the plate (1) on the heat block (4) as shown in Figure 2. As shown (Figure 2), in contrast to the conventional PCR plates, the walls of the wells (2) of the multi-well plate (1) do not project above the top surface of the block (4). The type of positioning provides several advantages: The pressure caused by the screw (12) to the lid (10) (heating element (11)) can be increased in order to obtain efficient sealing of the samples (9) sealed, for example, by a silicon mat (13). In this case the pressure is actually directed to those parts of the multiwell plate (1) which are supported by the top surface of the heat block (4) (or by parts of the top surface surrounding individual wells depending on the geometry of the heat block) and not to the thin walls of the wells of the plate as it is the case for the PCR tubes or conventional PCR plates. This advantage makes it possibe to increase the sealing pressure of the heated lid (10) several fold when compared to the conventionally used pressure of 30-50 g per well without cracking the conically shaped walls of the wells (2).

The extremely thin walls of the wells, i.e. 20-40 microns, are highly flexible as the multiwell plates are thermoformed out of highly elastic films (or sheets depending on the draw ratio). The walls of the wells are highly resistant against stress cracking, due to their flexibility and elasticity. As the wells of the plate, positioned on the heat block, are tightly sealed at room temperature, the air pressure in the wells will increase at elevated temperatures. The increased air pressure causes a deformation of the walls of wells and brings them in tight thermal contact with the surface of the walls of the individual sample wells machined in the body of the heat block. Standard PCR plates (having relatively thick and rigid walls of the wells) require that the conically shaped walls of the wells have to match perfectly with the shape of the wells machined in the body of the heat block to guarantee a close thermal contact (see for example U.S. Patent No 5,475,610). This requirement is not as critical for the ultrathin walled multiwell plates of the invention, due to flexibility and elasticity of the walls of the wells. Using this advantage, special shapes of both, the walls of the wells of the plate and the wells of the heat block can be differently designed. These differently designed wells can promote an even closer thermal contact after positioning the plate into the heat block.

Another aspect of the invention concerns the frame of the multiwell plates. As the plates can be formed of very thin films (depending on the draw ratio of the well; supra) the flexibility of, for example, standard-format plates, i.e. 96-well PCR (8,5 x 12,5 cm) plates, is such that handling is not easily possible anymore. Therefore, depending on the geometry of the plate, a supporting frame might be needed, for example for industry standard formats, i.e. 96-, 192-, 384-well PCR plates. This frame can support, for example in case of small plates, the edges of the plate, or individual wells of the plate, or groups of wells. For handling with robots, for example, the frame can be injection molded in the form of the standard skirted microplates containing the array of holes in the top surface of the frame matching the array of wells of the ultrathin multiwell plate. The plate can be attached to the frame by for example heat bonding. However, for small format plates including the frame can be formed as a single piece by using specially designed moulds.

The polypropylene-based plastics are PCR-compatible and therefore widely used for injection molding of PCR tubes and/or multiwell plates. In addition, they are resistant to stress cracking and have a reduced water vapor sorption when compared to other plastics (e.g. polycarbonate). Such plates can be thermoformed in both, standard industry formats, i.e. 96-, 192- and 384-well PCR plates for large scale applications, supported by robots and small foot-print formats to match small foot-print thermocyclers, i.e. "personal thermocyclers".

The following example serves to illustrate the invention but should not be construed as a limitation thereof.

Example:

Fig.1 illustrates a 36-well ultrathin walled multiwell plate according to the invention. The plate was designed for rapid temperature cycling of samples ranging from 0.5-4 µl using a small foot-print peltier-driven heat block thermocycler supplied with a "wine-press" type heated lid (Fig. 2). The volume of the wells is 16 µl and the distance between the wells is 4.5 mm, i.e. industry standard for high sample density 384-well PCR plates. The diameter of the openings of the wells is 3.8 mm and the height of the wells is 3 mm. The average thickness of the walls of the wells is 30 µm. The frame (3) was cut out of a polypropylene sheet of a thickness of 0.5 mm and heat bonded to the plate (1). The area of the plate (1)

is 30 x 30 mm. As shown in Figure 1, the handling of the plate (1) containing the multiple wells (2) is facilitated, by a rigid 0.5-1 mm thick plastic frame (3) which is heat bonded to the plate. As shown in Figure 2, the frame (3) is not in direct thermal contact with the block (4) during thermocycling because the inner contour (5) of the frame (3) matches the outer contour (6) of the heat block (4) of the thermocycler (7 = thermoelectric heat pump and 8 = air-forced heat sink).

The ultrathin walled multiwell plate according to the invention (Fig.1) was experimentally tested for the amplification of a 455-base pairs long fragment of human papilloma virus DNA. The sample volume was 3 µl. For various PCR reactions, the average ramping rate of the thermo cycler was varied from 4° C to 8° C per second. The samples (i.e. standard PCR-mixtures without any carrier molecules) were transferred into the wells of the plate by means of conventional pipetting equipment. The plate was covered by standard sealing film (Microseal A; MJ-Research, USA), transferred into the heatblock of the thermocycler and tightly sealed by the heated lid as shown in Fig. 2. Upon sealing, a number of 30 PCR cycles was performed in 15-25 minutes depending on the ramping rate of the thermo cycler. The PCR product was analyzed by conventional agarose electrophoresis. The 455-base pairs long DNA fragment was amplified with a high specificity at the indicated ramping rates (supra).

Plates according to the invention with well volumes of 35 µl were successfully tested for temperature cycling of samples of a volume of 20 µl. Thereby, 30 PCR cycles were performed in 20-30 minutes at a ramping rate of 6° C per second. Surprisingly, although the average thickness of the walls was 20 microns and the volume of the wells was 35 µl, samples of a volume of as few as 0.5 µl can be easily amplified without reducing the PCR efficiency.

In conclusion, the ultrathin walled multiwell plates according to the invention, allow a simple and rapid loading of multiple samples by conventional pipettes, rapid sealing of all samples by using conventional sealing films and rapid DNA amplification (15-30 minutes for 30 cycles) with an improved specificity typical for rapid cycling (Wittwer et al., Analytical Biochem., 186, 328-331 [1990]) using appropriate heat block thermocyclers (i.e. ramping rate in the range of 4° C to 8° C per second).

Claims

1. Ultrathin-walled multiwell plate for heat block thermocycling of samples comprising an array of small-volume wells of identical height with the similarly shaped sample wells formed in the top surface of the heat block of the thermocycler.

2. Ultrathin-walled multiwell plate according to claim 1, wherein the height of the wells of the plate is not more than the height of the sample wells formed in the top surface of the heat block of the thermocycler

3. Ultrathin-walled multiwell plate according to claim 1, wherein the walls of the wells are conically shaped.

4. Ultrathin-walled multiwell plate according to claim 1, wherein the thickness of the walls of the wells decreases from top to bottom.

5. Ultrathin-walled multiwell plate according to claim 1, wherein the wells of said multiwell plate are thermoformed into negative mould.

6. Ultrathin-walled multiwell plate according to claim 1, wherein the walls of the wells have an average thickness of 20-40 microns.

7. Ultrathin-walled multiwell plate according to claim 1, wherein the walls of the wells are deformable.

8. Ultrathin-walled multiwell plate according to claim 1, wherein the said microwell plate comprises a rigid supporting frame.

9. Ultrathin-walled multiwell plate according to claim 1, wherein the volume of the well is in the range of 16-85 µl.

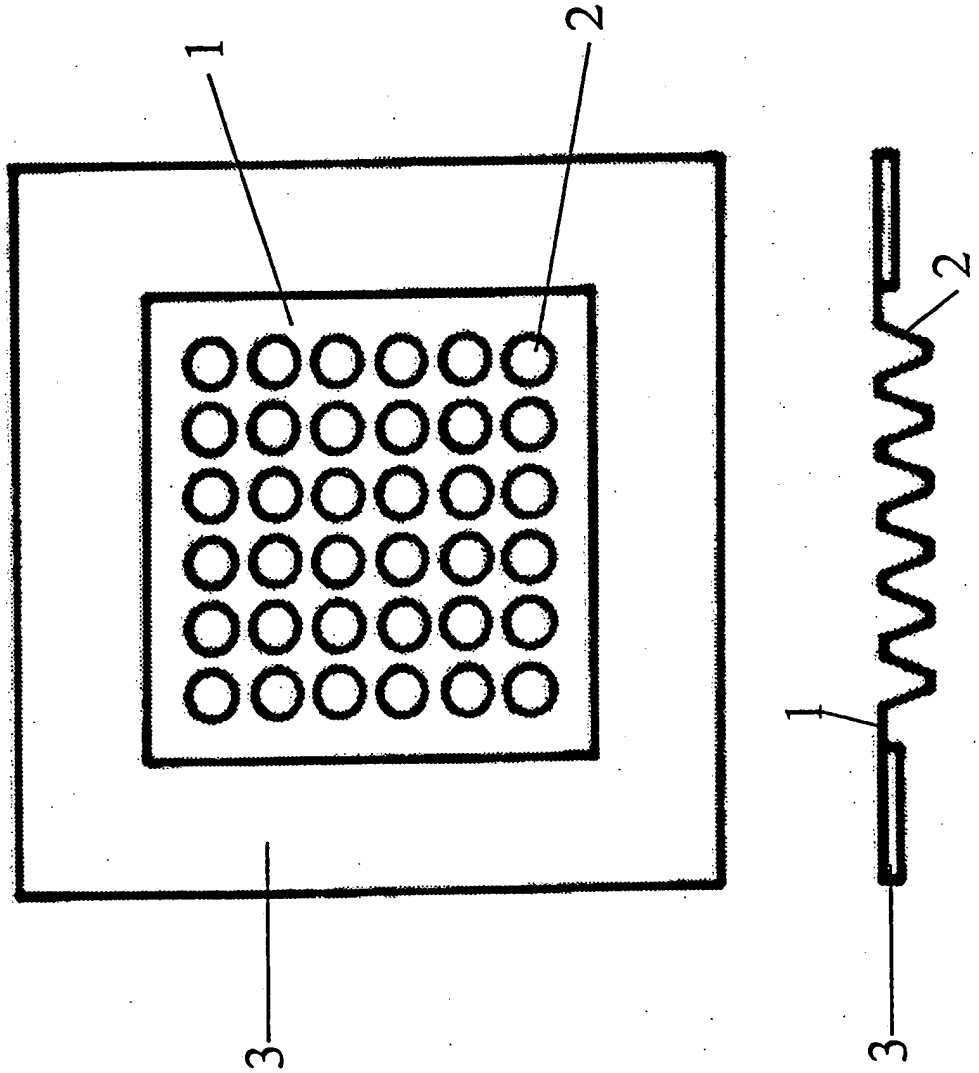

Fig. 1

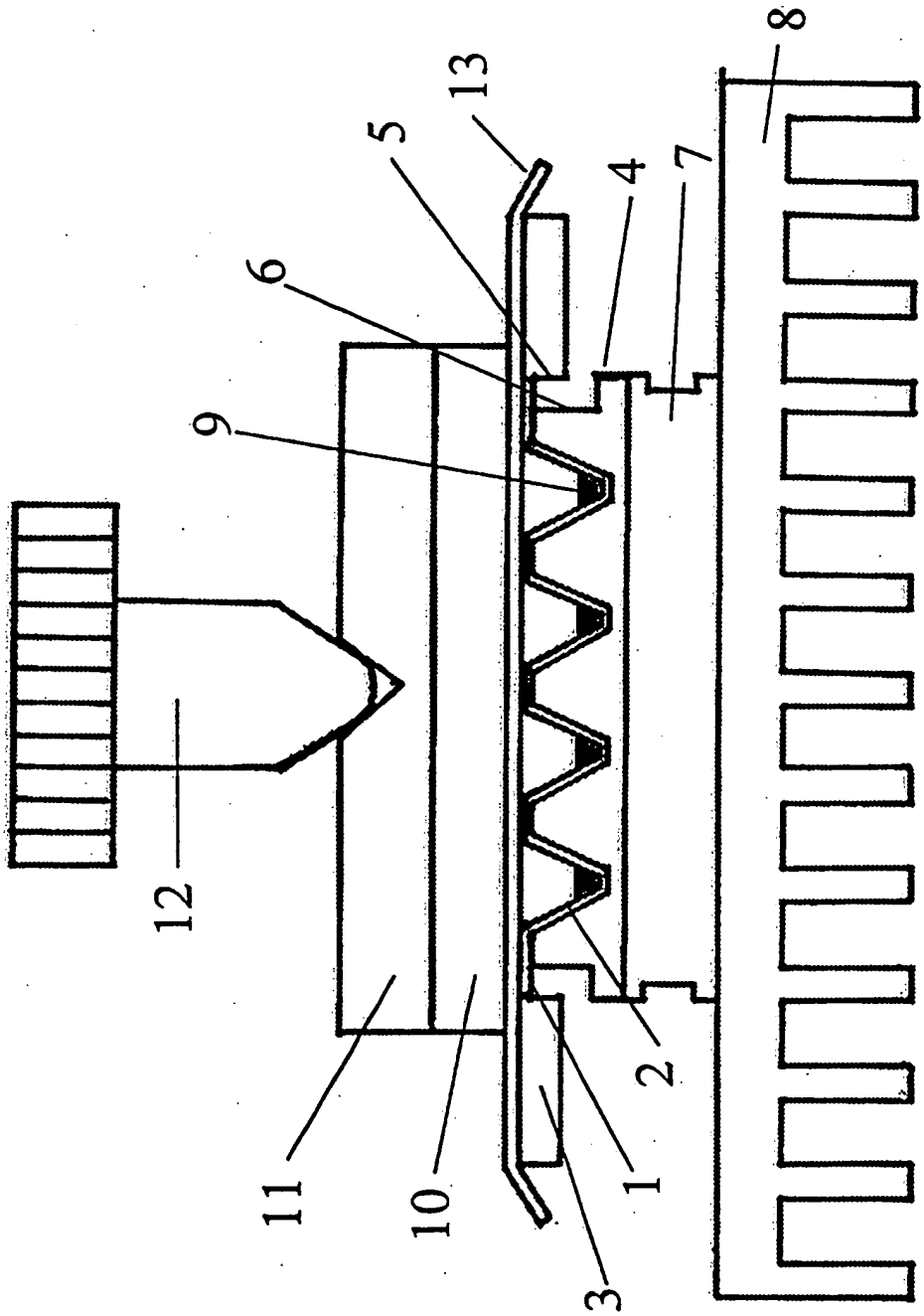

Fig. 2

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 B01L3/00 B01L7/00

According to International Patent Classification (IPC) or to both national classification and IPC

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	DE 40 22 792 A (MAX PLANCK GESELLSCHAFT) 6 February 1992 (1992-02-06) column 1, line 3 -column 1, line 17 column 1, line 31 -column 1, line 40 column 1, line 46 -column 1, line 52 column 1, line 64 -column 1, line 67 column 2, line 37 -column 2, line 50 column 2, line 65 -column 3, line 6 column 3, line 34 -column 3, line 67 column 4, line 15 -column 4, line 18	1,2,5, 7-9
A	column 4, line 29 -column 4, line 40 column 4, line 58 -column 4, line 68 column 7, line 8 -column 7, line 37	6
A	column 7, line 57 -column 7, line 62 column 16, line 56 -column 17, line 23 figures 1,2,10	4

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[X] Further documents are listed in the continuation of box C.

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PCT/EP 99/08178

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Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5 430 957 A (EIGEN MANFRED ET AL) 11 July 1995 (1995-07-11) column 1, line 28 -column 1, line 37 column 4, line 50 -column 4, line 53 column 4, line 62 -column 5, line 2 column 9, line 58 -column 9, line 64 column 10, line 62 -column 11, line 5 figure 5	1,2,8
X	US 5 601 141 A (GORDON STEVEN J ET AL) 11 February 1997 (1997-02-11) column 3, line 31 -column 3, line 45 column 3, line 67 -column 4, line 34 figures 1-4	1,8
X	WO 97 26993 A (BJS COMPANY LTD ;GUNTER IAN ALAN (GB)) 31 July 1997 (1997-07-31) page 1, line 23 -page 2, line 13 page 2, line 19 -page 2, line 24 page 4, line 11 -page 4, line 24 page 5, line 13 -page 5, line 16 figures 1,2	1-3,6,8
A	US 5 161 609 A (MARCADET-TROTON AGNES ET AL) 10 November 1992 (1992-11-10) column 1, line 12 -column 1, line 28 column 4, line 12 -column 4, line 25 column 5, line 64 -column 6, line 3 column 6, line 38 -column 6, line 68 figures 2,4	1,2

2

INTERNATIONAL SEARCH REPORT

Inte | ional Application No
PCT/EP 99/08178

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 4022792 A	06-02-1992	AT 125732 T	15-08-1995
		WO 9201513 A	06-02-1992
		DE 59106171 D	07-09-1995
		EP 0539369 A	05-05-1993
		JP 6500727 T	27-01-1994
US 5430957 A	11-07-1995	DE 4029004 C	02-04-1992
		AT 109382 T	15-08-1994
		WO 9204979 A	02-04-1992
		DE 59102453 D	08-09-1994
		EP 0548118 A	30-06-1993
		JP 6502580 T	24-03-1994
US 5601141 A	11-02-1997	NONE	
WO 9726993 A	31-07-1997	AU 1451097 A	20-08-1997
		EP 0876218 A	11-11-1998
US 5161609 A	10-11-1992	FR 2642156 A	27-07-1990
		AT 103062 T	15-04-1994
		AU 4963190 A	13-08-1990
		CA 2025465 A	21-07-1990
		DE 69007305 D	21-04-1994
		DE 69007305 T	29-09-1994
		EP 0379437 A	25-07-1990
		ES 2053128 T	16-07-1994
		WO 9008298 A	26-07-1990
		JP 3503445 T	01-08-1991